## Compositional Analysis of the Major Capsular Polysaccharides of Cryptococcus Neoformans by Highperformance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

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# COMPOSITIONAL ANALYSIS OF THE MAJOR CAPSULAR POLYSACCHARIDES OF *CRYPTOCOCCUS NEOFORMANS* BY HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION (HPAEC-PAD)

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## ABSTRACT

*Cryptococcus neoformans* is a predominantly saprophytic yeast that can cause serious infections, mostly in individuals with acquired immunodeficiency syndrome (AIDS).

A prominent virulence factor of *Cryptococcus neoformans* is its capsule. This is constituted mainly of glucans; the capsular polysaccharide, glucuronoxylomannan (GXM) (composed of mannose, xylose, glucuronic acid), and at least two minor carbohydrate antigens, galactoxylomannan (GalXM) and mannoproteins (MP).

In this paper we present an optimized HPAEC-based method for the rapid and effective determination of the complete carbohydrate constituents of GXM and GalXM.

*Cryptococcus neoformans* strain A 9759 serotype A and the acapsular mutant CAP 67 were grown in a totally dialyzable synthetic medium at 30°C. Culture supernatants were subjected to tangential filtration (10,000-$M_r$-cut-off) to separate high molecular weight cryptococcal products from medium constituents.

The supernatants were dialyzed against phosphate buffer saline (PBS) containing sodium azide (0.02%) and concentrated to a carbohydrate concentration of 1.5 mg/mL. GXM and GalXM were isolated and purified as described in the experimental section. For neutral sugar and uronic acid composition analysis, 2.5 mg polysaccharide fractions were hydrolyzed with 2 M trifluoroacetic acid (TFA, 100°C, 6 h), followed by lyophilization, then the residues were analyzed by HPAEC coupled with pulsed amperometric detection (PAD).

Separations were carried out using either a CarboPac PA 100 or a CarboPac PA 10 column and matching guard column (all from Dionex, Sunnyvale, CA, USA). Employing PA 100, as well as PA 10 column, baseline separation of the neutral monosaccharides galactose, glucose, xylose, mannose and glucuronic acid were obtained. Monosaccharide and uronic acid compositions of GXM and GalXM determined by this method were found to be highly accurate.

## INTRODUCTION

*Cryptococcus neoformans* is a predominantly saprophytic yeast that can cause serious infections, mostly in individuals with deficiencies in the immune system. This yeast is the major etiological agent of cryptococcosis which is the fourth most common cause of lethality in patients with the acquired immunodeficiency syndrome (AIDS).[1-2]

A prominent virulence factor of *C. neoformans* is its capsule. The major capsular antigen is a high molecular weight glucuronoxylomannan (GXM), that is an important virulence factor,[3] and also defines the organism's serotype

specificities.[4]  The biological and antigenic properties of GXM are related to the chemical structure of this major capsular polysaccharide antigen.  An additional capsular constituent is GalXM, which may also have important biological activities.[5]

Methods available for carbohydrate analysis of complex polysaccharides such as GXM and GalXM, are mainly based on determination of their constituent sugar residues obtained after chemical hydrolysis of the native polymers.  Procedures commonly applied for quantification of the liberated monosaccharides are hampered by the necessity of derivatization in gas chromatography, or inadequate separation and detection by HPLC.  A considerable improvement in the sensitivity of carbohydrate analysis was obtained by the development of high-performance anion-exchange chromatography (HPAEC) using pellicular ion exchange resins and pulsed amperometric detection (PAD) which has extremely high sensitivity for the carbohydrate separations.

The present study was undertaken to examine the usefulness of HPAEC-PAD for analysis of the carbohydrate constituents of GXM and GalXM.

## EXPERIMENTAL

### Equipment

The HPLC analyses were conducted using a Dionex (Sunnyvale, CA, USA) Model 4000i, quaternary advanced gradient pump, equipped with a Dionex pulsed electrochemical detector (PED) with a gold working electrode and a silver-silver chloride reference electrode.  The program for PED in the pulsed amperometric mode was $E_1 = 0.10$ V, $t_1 = 0\text{-}500$ ms, integration period 200 ms (beginning at 300 ms), $E_2 = 0.60$ V, $t_2 = 510\text{-}590$ ms; $E_3 = -0.60$ V, $t_3$ 600-650 ms.  A Dionex DXP single-piston pump was used to add 0.5 M sodium hydroxide to the column effluent through a tee connection at 1 mL/min before the cell of the detector during elution.  Also, from Dionex, were CarboPac PA 100 and CarboPac Pa 10 anion-exchange columns (both 250 x 4.0 mm I.D.) with associated guard column.

Samples were injected using a Rheodyne Model 9125 non-metal (PEEK) injection valve with a PEEK sample loop of 10 μL (Cotati, CA, USA).  The Dionex eluent degas module was employed to sparge and pressurize the eluent with helium.  Data collection was performed on an Epson-PC AX3 computer

with an advanced computer interface (Dionex) and AI-450 software (Dionex). The AI-450 software also controlled the Dionex instrument and the gradient program.

## Reagents

DL-arabinose, D-(+)-glucose, D-(+)-galactose, D-(+)-mannose, D-(+)xylose, and D-glucuronic acid, as standards for analysis, and other reagents were purchased from Sigma (Milan, Italy), unless otherwise specified. Fifty percent (w/w) sodium hydroxide solution and anhydrous sodium acetate were obtained from J.T. Baker (Milan, Italy). HPLC-grade water was from Carlo Erba (Milan, Italy).

Non-serotype specific monoclonal antibody directed against GXM was a kind gift from Dr. A. Casadevall (Albert Einstein College of Medicine, Yeshiva University, Bronx, N.Y., USA). Encapsulated *C. neoformans* strain A 9759, serotype A, was kindly provided by Dr. E. Reiss Centre for Disease Control, Atlanta, GA, USA). Strain CAP 67, an acapsular mutant, was provided by Dr. E. Jacobson (Richmond, VA, USA).

## Cryptococcal Cultures

Strain A 9759 (GXM) and CAP 67 (GalXM and MP) were transferred from sabouraud agar plates to starter cultures consisting of 100 mL of dialyzable chemically defined medium,[6] and then isolated and purified as previously described.[7] Briefly, starter cultures were incubated at 30°C for 48 h under agitation and transferred to 2 liter aliquots of the same medium in Erlenmeyer flasks.

After culturing under agitation for 4 days, late log cultures were pooled and yeast separated from supernatants with a Millipore Pellicon tangential flow system equipped with 0.2 micron filter cassettes (Millipore, Milan, Italy). Supernatants were dialyzed against phosphate buffer saline (PBS, 0.01 M, pH 7.2, 0.15 M NaCl) containing 0.02 M sodium azide and concentrated 20 fold using Pellicon cassettes with a 10,000 daltons exclusion limit.

Concentrated supernatants were heat-inactivated (56°C for 30 min) and supplemented with 25 µg/mL of phenyl-methyl-sulfonyl fluoride to inactivate lytic enzymes possibly present in these preparations.

## Cell Wall Preparation

Carbohydrate concentration of cryptococcal cultures supernatants was adjusted to 1.5 mg/mL as measured by the phenol sulfuric acid method[8] before gel filtration. This was performed using a Sepharose CL-4B column (90 x 5 cm I.D., Pharmacia, Milan, Italy) at a flow rate of 1.5 mL/min in PBS/sodium azide. Ten mL fractions were collected and analyzed for carbohydrate and protein content by the phenol sulfuric acid method and absorbance at 280 nm, respectively. Carbohydrate-containing fractions were pooled and precipitated with 90% ethanol. GXM was considered of sufficient purity by native polyacrylamide gel electrophoresis using overloaded samples.[9] GalXM was obtained from CAP 67 supernatants by affinity Chromatography on ConA Sepharose-6B (Pharmacia), exactly as decribed by Dong et al.[10] Purity of GalXM was confirmed by PAGE analysis of overloaded gel.

## Acid Hydrolysis

Samples (2.5 mg) of GXM or GalXM were hydrolyzed to their constituent monosaccharides in 2 M trifluoroacetic acid at 100°C for 6 h. The hydrolyzates were dried by lyophilization and redissolved in water containing arabinose as internal standard, immediately prior to analysis.

## Procedures for Quantitative Analysis

A carbohydrate stock solution was prepared by dissolving in water the appropriate amounts of galactose, glucose, xylose, mannose, and glucuronic acid. A 1.25 mg/mL internal standard stock solution was prepared by dissolving arabinose in water. Appropriate amounts of the stock solution were diluted with water to produce working standard solutions at five different concentrations within the required range. An appropriate volume of internal standard solution was added to each solution to give a concentration of 0.125 mg/mL of arabinose. Calibration graphs were plotted based on the linear regression analysis of the peak-area ratios. Triplicate determinations were performed for each sample and the results were reported as the average.

## RESULTS AND DISCUSSION

Carbohydrate analysis by HPAEC-PAD is usually performed in an alkaline medium, using sodium hydroxide as mobile phase. Depending on the

NaOH concentration, all important monosaccharides can be completely separated. However, xylose is poorly separated from mannose even when dilute sodium hydroxide solutions are used. These elution patterns are obviously not suitable to establish the monosaccharide profile of GXM, in which xylose and mannose are simultaneously present. Furthermore, we were interested in developing a method capable of separating galactose from the above carbohydrates. Indeed, galactose is a major component of galactoxylomannan, a minor soluble antigen associated with the cell envelope of *C. neoformans*.[5]

In order to select the optimum column and chromatographic conditions to perform rapid and reproducible separation and quantitation of the above monosaccharides, the effect of mobile phase composition on retention and selectivity was examined using two different commercial pellicular anion-exchange columns marketed by Dionex as CarboPac PA 10 and CarboPac PA 100. The first column is packed with a new anion-exchanger specifically designed for the quantification of acidic, neutral, and amino monosaccharides, especially those derived from glycoconjugates, whereas, the CarboPac PA 100 is commonly used for oligosaccharide separations. However, the suitability of the CarboPac PA 100 column for monosaccharide separation has also been demonstrated.[11-12] In a first experiment, the two columns were evaluated by eluting galactose, glucose, xylose, and mannose under isocratic conditions with mobile phases containing sodium hydroxide at different concentrations ranging from 3 to 15 mM. However, with both columns, the incomplete separation of xylose and mannose was achieved.

Using the CarboPac PA100 column, we completely separated galactose, glucose, xylose, and mannose by elution with pure water at a flow rate of 1 mL/min. Performing isocratic separation with water, post column addition of 0.5 M NaOH was necessary to maintain optimum detector sensitivity and minimize baseline drift. Under these conditions mannose eluted as a rather broad peak. To improve the separation of mannose from the other monosaccharides, a linear gradient step from pure water to 150 mM sodium acetate in 120 mM sodium hydroxide over 7 min was carried out after 25 min of isocratic elution.

Elution of glucuronic acid requires much stronger conditions than neutral monosaccharide mannose. Elution of glucuronic acid was achieved by a second linear gradient step in which the proportion of sodium acetate was increased from 150 mM to 200 mM over 6 min. Then the concentration of sodium acetate was ramped to 250 mM, followed by a 10 min elution with 300 mM NaOH to regenerate the column. Column equilibration to the start conditions was obtained eluting with water 15 min before injection.
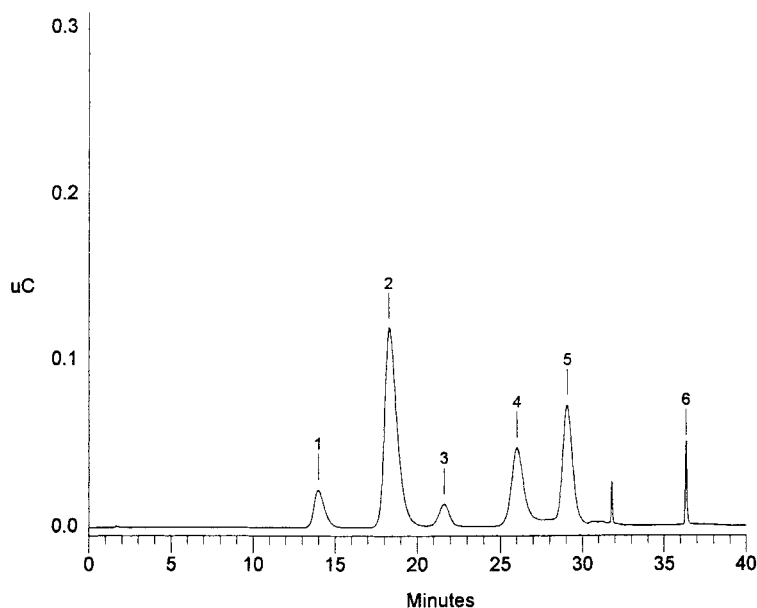
**Figure 1.** Separation on a CarboPac PA100 column of a standard mixture containing 1) arabinose (0.75 μmol/L), 2) galactose (2.75 μmol/L), 3) glucose (0.44 μmol/L), 4) xylose (1.75 μmol/L), 5) mannose 1.60 μmol/L), 6) glucuronic acid (0.45 μmol/L). Chromatographic conditions as in the text.

Similar results were obtained using the CarboPac PA10 column. However, using this column, the monosaccharide components were separated in less than 27 min by a single isocratic step using water at a flow rate of 1.2 mL/min. Then, glucuronic acid was eluted by application of 12 min linear gradient to 150 mm NaOH and 125 mM sodium acetate.

Including column regeneration and equilibration to the start conditions, total running time was 64 min and 69 min using column CarboPac PA 100 and column CarboPac PA 10, respectively. However, these protocols allow for the analysis of neutral monosaccharides and glucuronic acid in one single run.

The quantification of the monosaccharides and glucuronic acid was performed by an internal standard method. Arabinose was selected as the internal standard because it is not naturally present in both GXM and GalXM hydrolyzates, is completely resolved in the chromatogram from the other

## Table 1

### Retention Time, Standard Deviation, and Relative Standard Deviation of a Standard Solution of Carbohydrates on CarboPac Columns
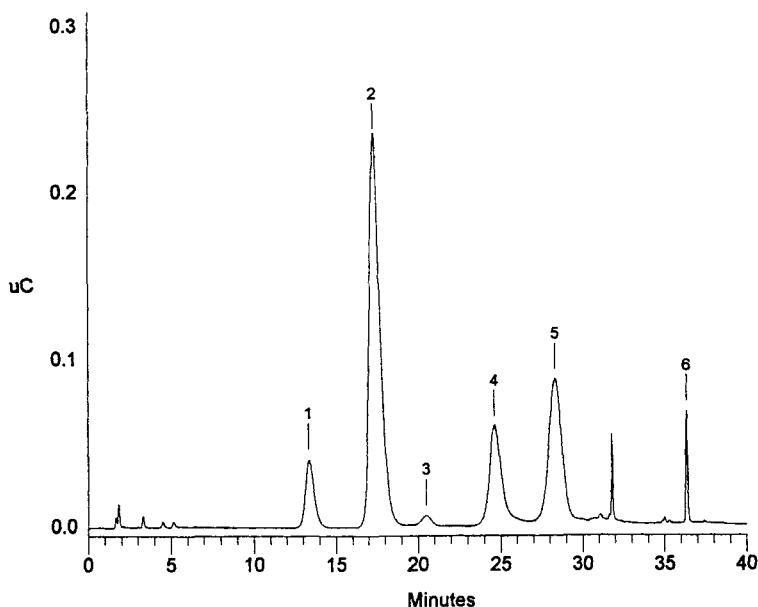
| | Column | | | | | |
|---|---|---|---|---|---|---|
| | CarboPac PA10 | | | CarboPac PA100 | | |
| Sample | tr Mean (min) | S.D. (min) | R.S.D. % | tr Mean (min) | S.D. (min) | R.S.D. % |
| Arabinose | 13.18 | 0.218 | 1.65 | 13.30 | 0.311 | 2.39 |
| Galactose | 16.44 | 0.257 | 1.57 | 17.80 | 0.403 | 2.26 |
| Glucose | 20.01 | 0.489 | 2.44 | 20.35 | 0.511 | 2.51 |
| Xylose | 23.93 | 0.492 | 2.06 | 25.17 | 0.614 | 2.44 |
| Mannose | 26.59 | 0.691 | 2.60 | 28.45 | 0.478 | 1.68 |
| Glucuronic Acid | 42.39 | 0.114 | 0.27 | 36.31 | 0.103 | 0.28 |

*tr (mean value, n=12) = Retention Time, S.D. = Standard Deviation, R.S.D. = Relative Standard Deviation.
Chromatographic Conditions as in the Text.

carbohydrates, and is eluted near the peaks of interest. A typical chromatogram of galactose, glucose, xylose, mannose, and glucuronic acid with the internal standard arabinose obtained with the CarboPac PA100 column, is shown in Fig. 1. The reproducibility of the retention times under the optimized chromatographic conditions was assessed for both columns by carrying out replicate analyses (n=12) of a known monosaccharides and glucuronic acid mixture. Results are summarized in Table 1 and show that with both columns the relative standard deviations were better than 2.65% for nutral sugars and did not exceed 0.30% for uronic acid which eluted under gradient conditions.

The calibration graphs for all compounds of interest, obtained by the peak-area ratio method, showed excellent linearity over the concentration range of 30-300 μg/mL (75-750 μg/mL for galactose), with correlation coefficients better than 0.998, and nearly passed through the origin. The minimum detectable limits, calculated as the concentration which gave peak height three times greater than the baseline noise level in the assay, were estimated to be 10 ng for xylose, mannose, and glucuronic acid and 5 ng for galactose and glucose, respectively.

**Figure 2.** Typical chromatogram of hydrolyzed GalXM obtained with the CarboPac PA100 column under the chromatographic conditions reported in the text. Peak identification: 1) arabinose (internal standard), 2) galactose, 3) glucose, 4) xylose, 5) mannose, 6) glucuronic acid.

A typical chromatogram obtained for GalXM hydrolyzed, using a PA100 column under the conditions described above, is reported in Fig 2. The major carbohydrate residues present in GalXM were galactose, xylose, mannose, and glucuronic acid. Substantially similar chromatographic profile was observed in the hydrolyzed GXM, but as expected, galactose was not detected. Moreover, in all the composition analyses, traces of glucose were found and this was presumed to be due to small quantities of starch-like glucan, a known contaminant in *C. neoformans* culture filtrates.[13]

Molar ratios of the substituent sugars, determined by HPAEC-PAD, were calculated relative to mannose, taken as 3.0 and are reported in Table 2. No substituents other than those indicated in Table 2 were identified and similar results were obtained using both columns.

## Table 2

### Carbohydrate Composition of GXM and GalXM

| Sample | Galactose | Molar Ratio | | |
| | | Xylose | Mannose | Uronic Acid |
|--------|-----------|--------|---------|-------------|
| GXM | n.d* | 1.10 | 3.00 | 0.83 |
| GalXM | 5.2 | 1.97 | 3.00 | 0.16 |

*not determined.

In conclusion we have shown that the high resolving capacity of the anion-exchange pellicular HPLC columns, CarboPac PA 100 and CarboPac PA 10, combined with the ease and sensibility of PAD detection, provide a valuable methodology for the complete compositional analysis of GXM and GalXM polysaccharides in one single chromatographic run.

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